SUPPLEMENTAL MATERIALS

NAD^+ Repletion Reverses Heart Failure with Preserved Ejection Fraction

Dan Tong[1], Gabriele G. Schiattarella[1], Nan Jiang[1], Francisco Altamirano[1], Pamela A. Szweda[1], Abdallah Elnwasany[1], Dong Ik Lee[2], Heesoo Yoo[1], David A. Kass[2], Luke I. Szweda[1], Sergio Lavandero[1,3], Eric Verdin[4], Thomas G. Gillette[1], and Joseph A. Hill[1,5,*]

Short Title: NAD^+ repletion reverses HFpEF

[1] Department of Internal Medicine (Cardiology), University of Texas Southwestern Medical Center, Dallas, Texas, USA, 75390-8573

[2] Department of Medicine (Cardiology), Johns Hopkins School of Medicine, Baltimore, Maryland.

[3] Advanced Center for Chronic Diseases (ACCDiS) & Corporacion Estudios Cientificos de las Enfermedades Cronicas (CECEC), Faculty of Chemical & Pharmaceutical Sciences & Faculty of Medicine, University of Chile, Santiago 8380492, Chile

[4] Bulk Institute for Research on Aging, Novato, CA, USA, 94945

[5] Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, Texas, USA, 75390-8573

***Correspondence:** Joseph A. Hill, M.D., Ph.D. Division of Cardiology, University of Texas Southwestern Medical Center. 6000 Harry Hines Blvd, NB11.200. Dallas, Texas, 75390-8573
E-mail: joseph.hill@utsouthwestern.edu

Online Figure I. Phenotype of HFpEF Mice and EM Quantification

Animals were fed with regular Chow vs HFD+L-NAME for 5 weeks (HFpEF).

(A) Body weight (BW)

(B) Systolic blood pressure (SBP)

(C) Diastolic blood pressure (DBP)

(D) Area under the curve (AUC) of glucose tolerance test

(E) LV ejection fraction

(F) Heart weight (HW) to tibia length (TL) ratio

(G) E/E' by tissue Doppler echocardiography

(H) Running distance during exercise exhaustion test

(I) Lung water to TL ratio between control (Chow) and HFpEF mice.

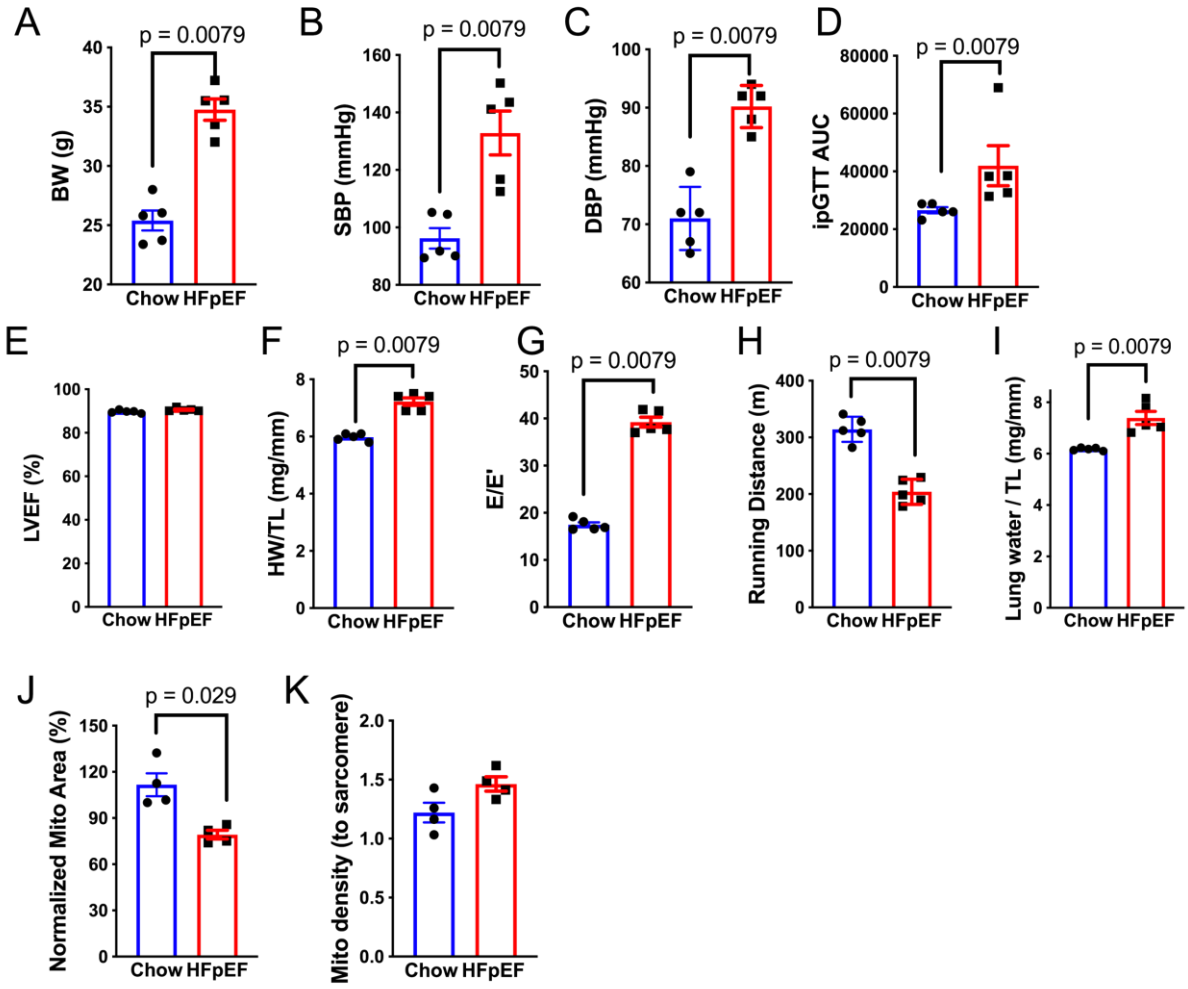
Results are presented as mean ± SEM, N=5 mice in each group.

(J) Quantification of mitochondria area in EM by Image J software. Values were normalized to Chow group. N = 4 mice in each group, 100 mitochondria counted for each mouse.

(K) Quantification of mitochondrial density as demonstrated by the ratio of mitochondria number to sarcomere number in a given field. N = 270 and 327 mitochondria from 4 mice in each group (Chow vs HFpEF).

Mann-Whitney test. P values listed on each bar graph.

Online Fig I


A
p = 0.0079
BW (g)
40
35
30
25
20
Chow HFpEF
B
p = 0.0079
SBP (mmHg)
160
140
120
100
80
Chow HFpEF
C
p = 0.0079
DBP (mmHg)
100
90
80
70
60
Chow HFpEF
D
p = 0.0079
ipGTT AUC
80000
60000
40000
20000
0
Chow HFpEF
E
LVEF (%)
100
80
60
40
20
0
Chow HFpEF
F
p = 0.0079
HW/TL (mg/mm)
8
6
4
2
0
Chow HFpEF
G
p = 0.0079
E/E'
50
40
30
20
10
0
Chow HFpEF
H
p = 0.0079
Running Distance (m)
400
300
200
100
0
Chow HFpEF
I
p = 0.0079
Lung water / TL (mg/mm)
8
6
4
2
0
Chow HFpEF
J
p = 0.029
Normalized Mito Area (%)
150
120
90
60
30
0
Chow HFpEF
K
Mito density (to sarcomere)
2.0
1.5
1.0
0.5
0.0
Chow HFpEF

Online Figure II. Mitochondrial Protein Expression in HFpEF Mouse Hearts

(A) Representative immunoblot image depicting the levels of electron transport chain (ETC) complex I-V in mice exposed to Chow, HFpEF (HFD+L-NAME), HFD alone, and L-NAME alone.

(B) NADH dehydrogenase activity measured in mitochondria isolated from hearts of different treatment groups. N=4 for each group. Kruskal-Wallis test with Dunn's multiple comparisons test.

(C) Representative immunoblot depicting protein abundances of VLCAD, LCAD, MCAD, HADHA, PDHE1α, VDAC, and Tom20 in the mitochondrial fraction isolated from LV of different treatment groups.

(D) Densitometric analysis ratio between various proteins relative to VDAC. Values were normalized to Chow group. N = 8 for VLCAD and LCAD in group chow and HFpEF. N=4 for all other groups. Kruskal-Wallis test with Dunn's multiple comparisons test.

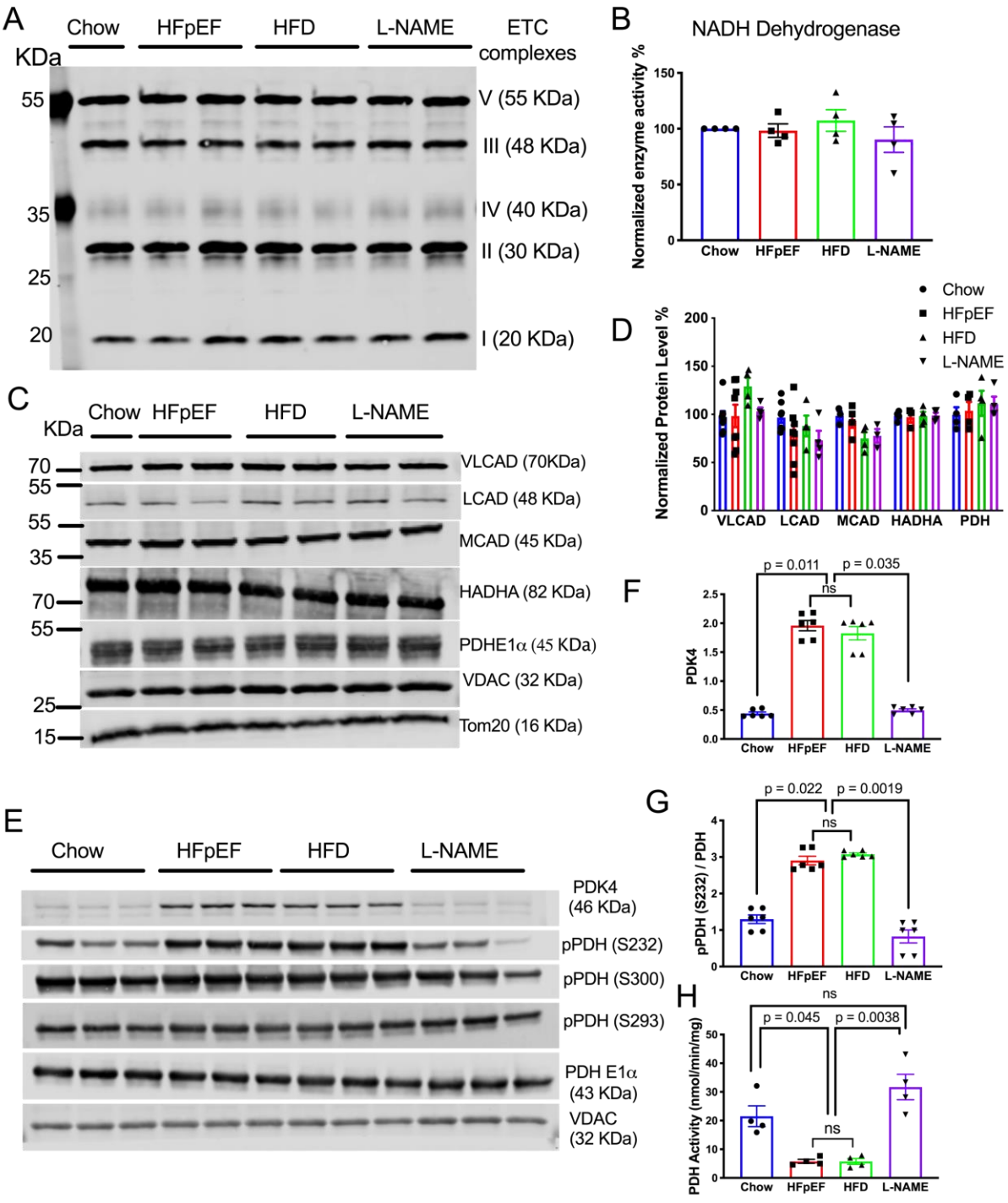
(E) Representative immunoblot depicting protein abundances of PDK4, PDHE1α, phosphorylated PDHE1α on serine 232, 300, and 293 sites and VDAC in mitochondrial fraction isolated from LV of different treatment groups.

(F) Densitometric analysis ratio of PDK4 relative to VDAC. N=6 mice in each group. Kruskal-Wallis test with Dunn's multiple comparisons test.

(G) Densitometric analysis ratio between phosphorylated PDHE1α serine 232 to VDAC. N=6 mice in each group. Kruskal-Wallis test with Dunn's multiple comparisons test.

(H) PDH activity measured in mitochondria freshly isolated from LVs of different treatment groups. N=4 mice in each group. Kruskal-Wallis test with Dunn's multiple comparisons test.

Online Fig II



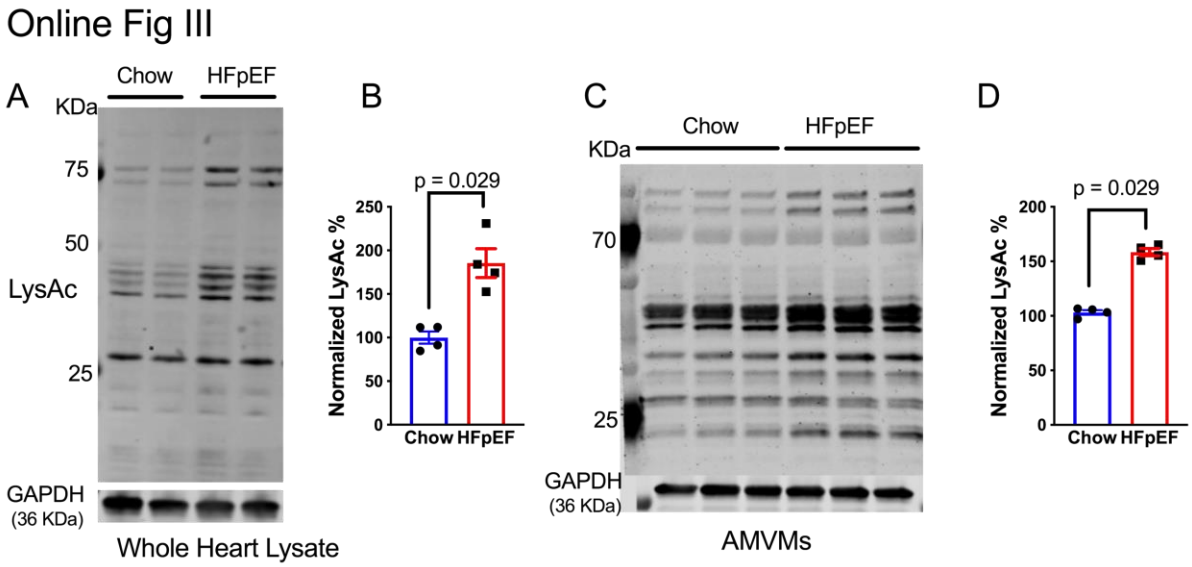
Online Figure III. Protein Hyperacetylation in HFpEF Hearts

(A) Representative immunoblot image depicting acetylated protein and GAPDH in LV from control and HFpEF mice.

(B) Densitometric analysis ratio of total acetylated protein relative to GAPDH. N=4 mice for each group. Mann-Whitney test.

(C) Representative immunoblot image depicting acetylated protein and GAPDH expression in adult mouse cardiomyocytes (AMVMs) isolated from hearts of control and HFpEF mice.

(D) Densitometric analysis ratio of total acetylated protein relative to GAPDH. N=4 mice for each group. Mann-Whitney test.



Online Figure IV. Cardiomyocyte-specific *Sirt3* KO mice

(A) Representative immunoblot image depicting protein abundances of Sirt3 in heart and liver tissue isolated from Sirt3 KO (αMHC-Cre; Sirt3$^{fl/fl}$) and control mice (αMHC-Cre and Sirt3$^{fl/fl}$).

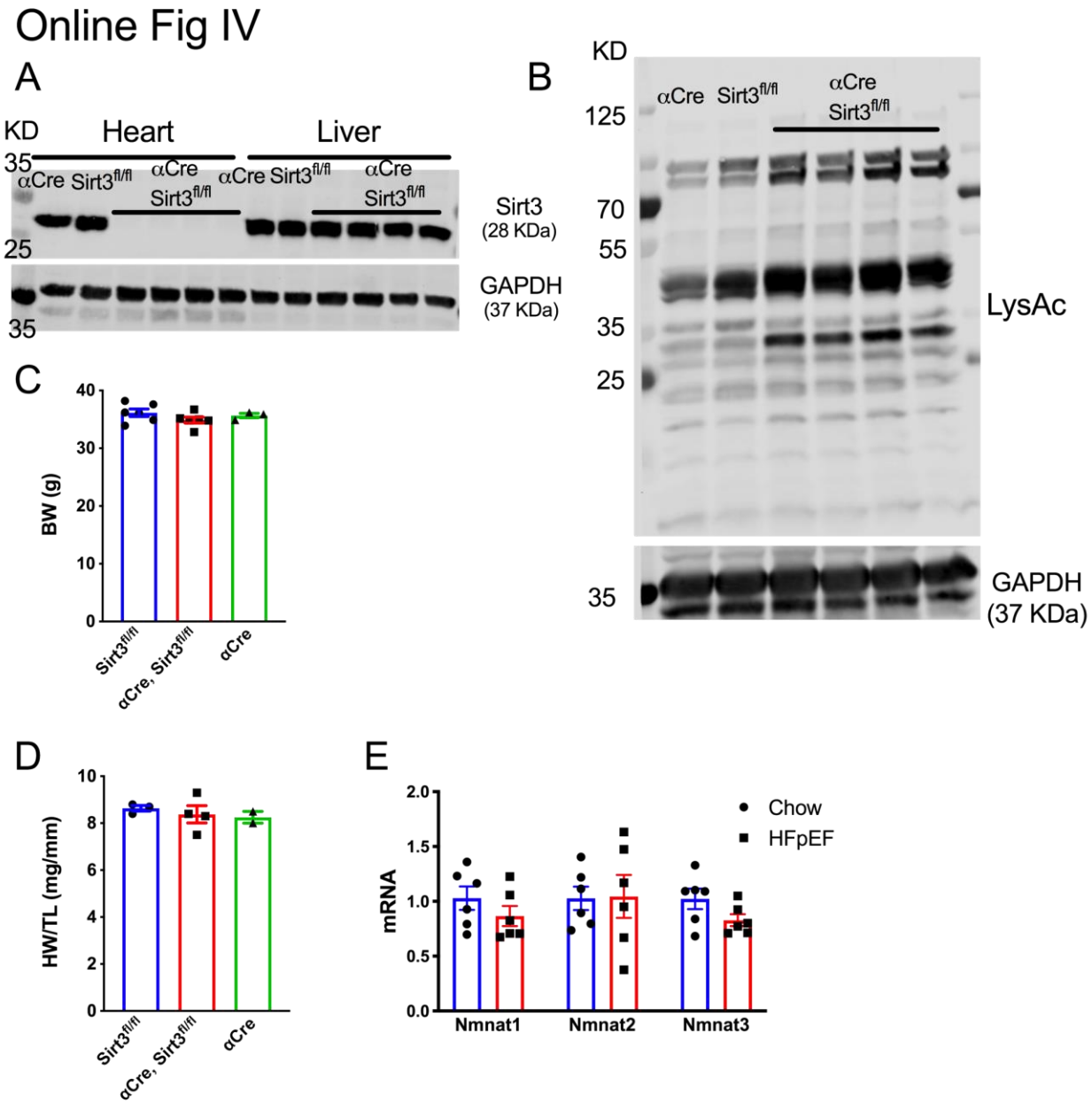
(B) Representative immunoblot image depicting acetylated protein in hearts isolated from Sirt3 KO (αMHC-Cre; Sirt3$^{fl/fl}$) and control mice (αMHC-Cre and Sirt3$^{fl/fl}$).

(C) Body weight (BW), N=6, 4, 3 in each group

(D) Heart weight to tibia length ratio (HW/TL), N= 3, 4, 2 in each group

Of the above three groups. Kruskal-Wallis test.

(E) Nmnat1, 2, 3 mRNA level in LV tissue of control and HFpEF mice. N=6 mice in each group. Mann-Whitney test.



Online Figure V. Effect of Nicotinamide Riboside (NR) Treatment

(A) Representative immunoblot image depicting protein levels of acetylated protein, Sirt1 and Sirt3 in LV of mice fed regular Chow (Chow) and HFpEF mice treated with placebo (HFpEF+PL) vs NR (HFpEF+NR).

(B) Densitometric analysis ratio of various proteins relative to GAPDH. Values were normalized to Chow group. N=4 mice for each group. Kruskal-Wallis test with Dunn's multiple comparisons test.

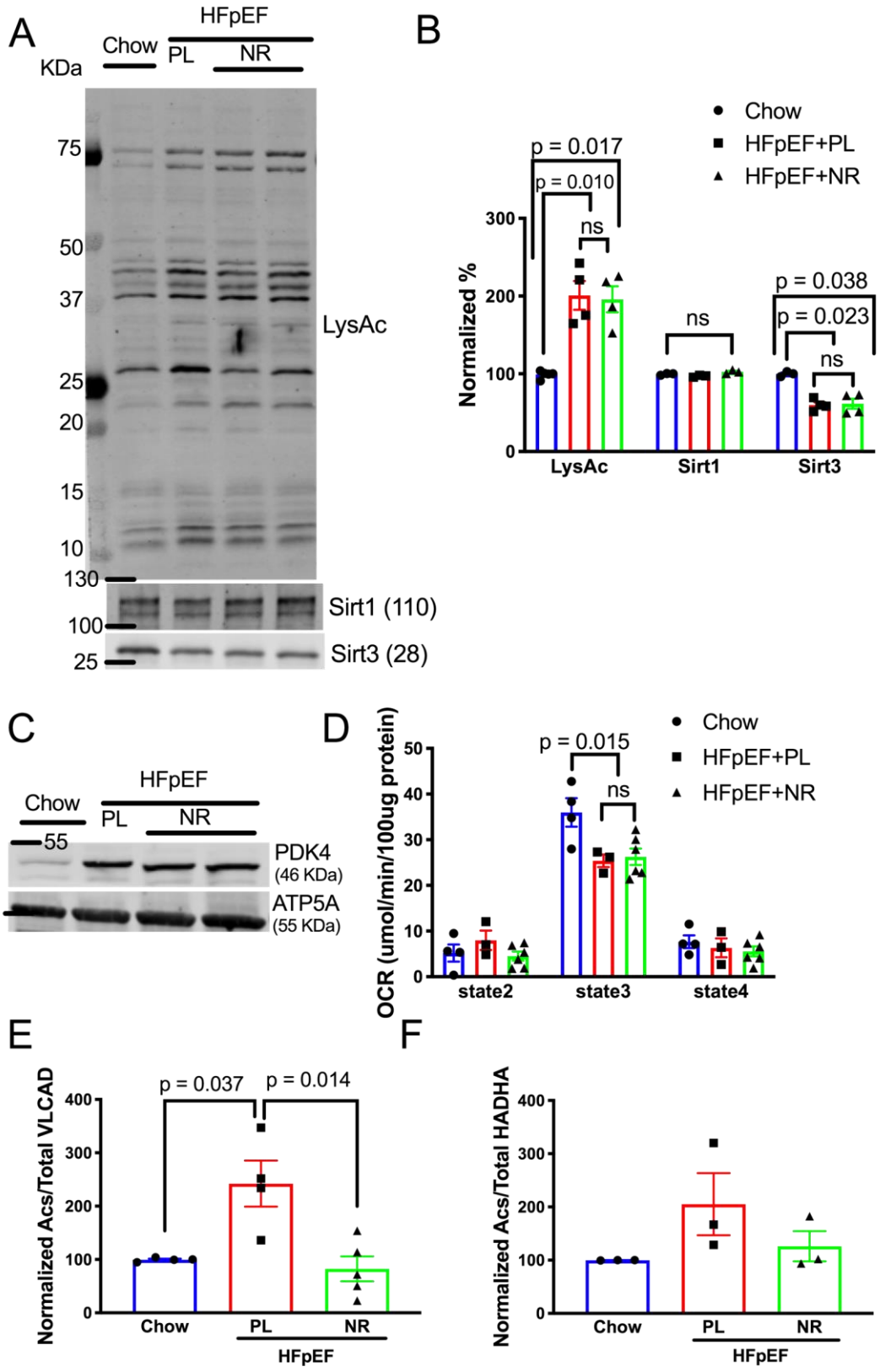
(C) Representative immunoblot image depicting protein abundances of PDK4 and ATP5A in LV of various treatment groups.

(D) OCR of isolated mitochondria exposed to pyruvate as substrate from various treatment groups. N=4 mice for each group. Two-way ANOVA followed by Sidak's multiple comparisons test.

(E) Densitometric analysis ratio of acetylated to total VLCAD. Values were normalized to Chow group. N=4 mice for chow and PL groups, N=5 for NR group. Kruskal-Wallis test with Dunn's multiple comparisons test.

(F) Densitometric analysis ratio of acetylated relative to total HADHA. N=3 mice for each group. Kruskal-Wallis test with Dunn's multiple comparisons test.

Online Fig V


A
HFpEF
Chow
PL
NR
KDa
75
50
37
25
20
15
10
130
100
25
LysAc
Sirt1 (110)
Sirt3 (28)
B
Chow
HFpEF+PL
HFpEF+NR
Normalized %
p = 0.017
p = 0.010
ns
ns
p = 0.038
p = 0.023
ns
LysAc
Sirt1
Sirt3
C
HFpEF
Chow
PL
NR
55
PDK4
(46 KDa)
ATP5A
(55 KDa)
D
Chow
HFpEF+PL
HFpEF+NR
OCR (umol/min/100ug protein)
p = 0.015
ns
state2
state3
state4
E
Normalized Acs/Total VLCAD
p = 0.037
p = 0.014
Chow
PL
NR
HFpEF
F
Normalized Acs/Total HADHA
Chow
PL
NR
HFpEF

Online Figure VI. Phenotype of HFpEF Mice Treated with Nicotinamide Riboside (NR)

Mice fed with Chow (Chow) and HFpEF mice treated with placebo (HFpEF+Pl) vs NR (HFpEF+NR).

(A) Body weight. N=9, 10, 15 mice per group. One-way ANOVA followed by Tukey's multiple comparisons test.

(B) Daily food intake. N=5 mice per group.

(C) Systolic blood pressure (SBP). N=5 mice per group.

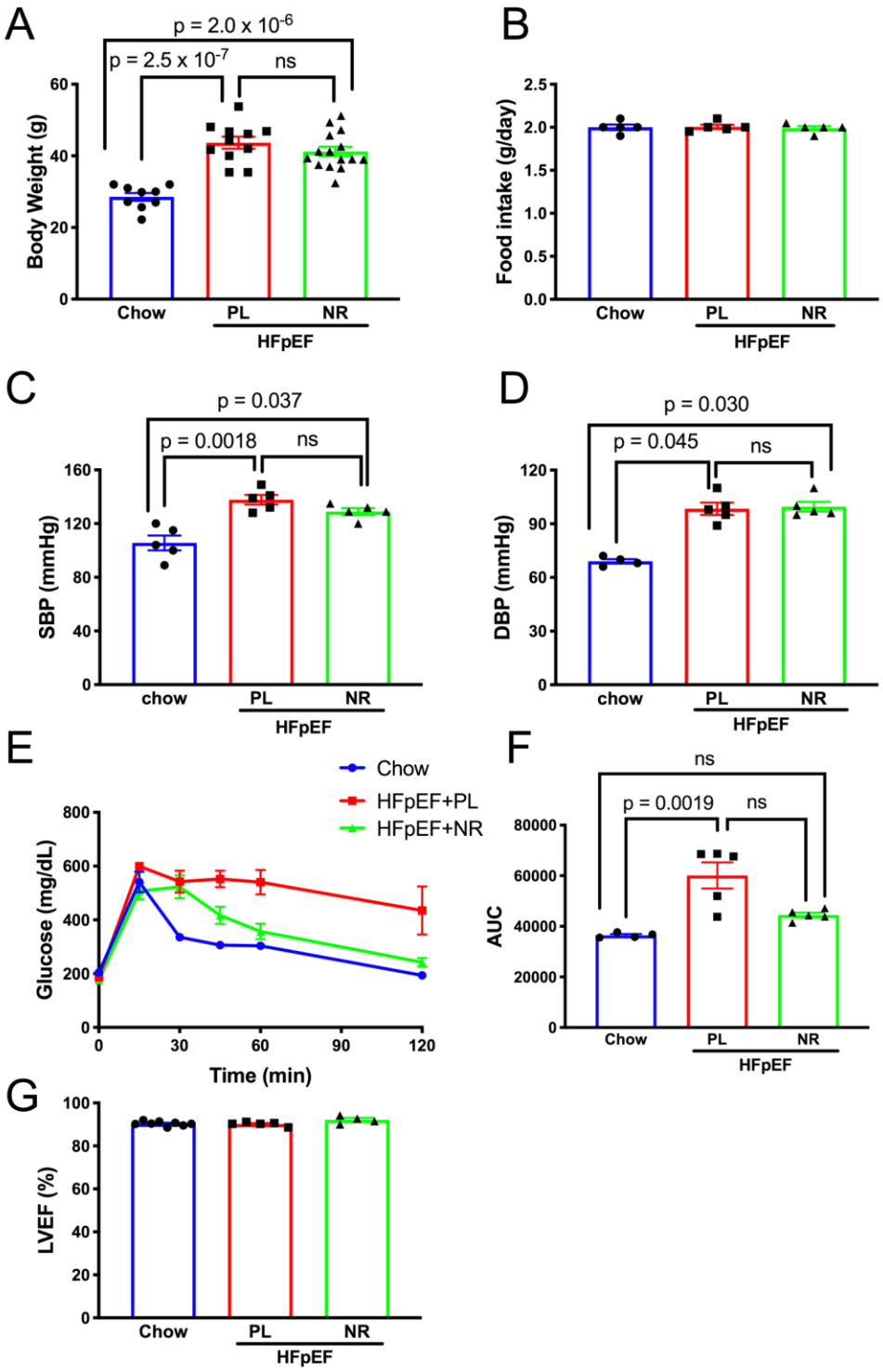
(D) Diastolic blood pressure (DBP). N=5 mice per group.

(E) Glucose levels during ipGTT test. N=5 mice per group.

(F) Area under the curve (AUC) of glucose tolerance test. N=5 mice per group. Results of Kruskal-Wallis test with Dunn's multiple comparisons test depicted in figure (results of one-way ANOVA followed by Tukey's multiple comparisons test: Chow vs HFpEF+Pl, p=0.0011; HFpEF+Pl vs HFpEF+NR, p=0.013; Chow vs HFpEF+NR, p=0.25)

(G) LV ejection fraction (LVEF). N=5 mice per group. Kruskal-Wallis test with Dunn's multiple comparisons test for B-G.

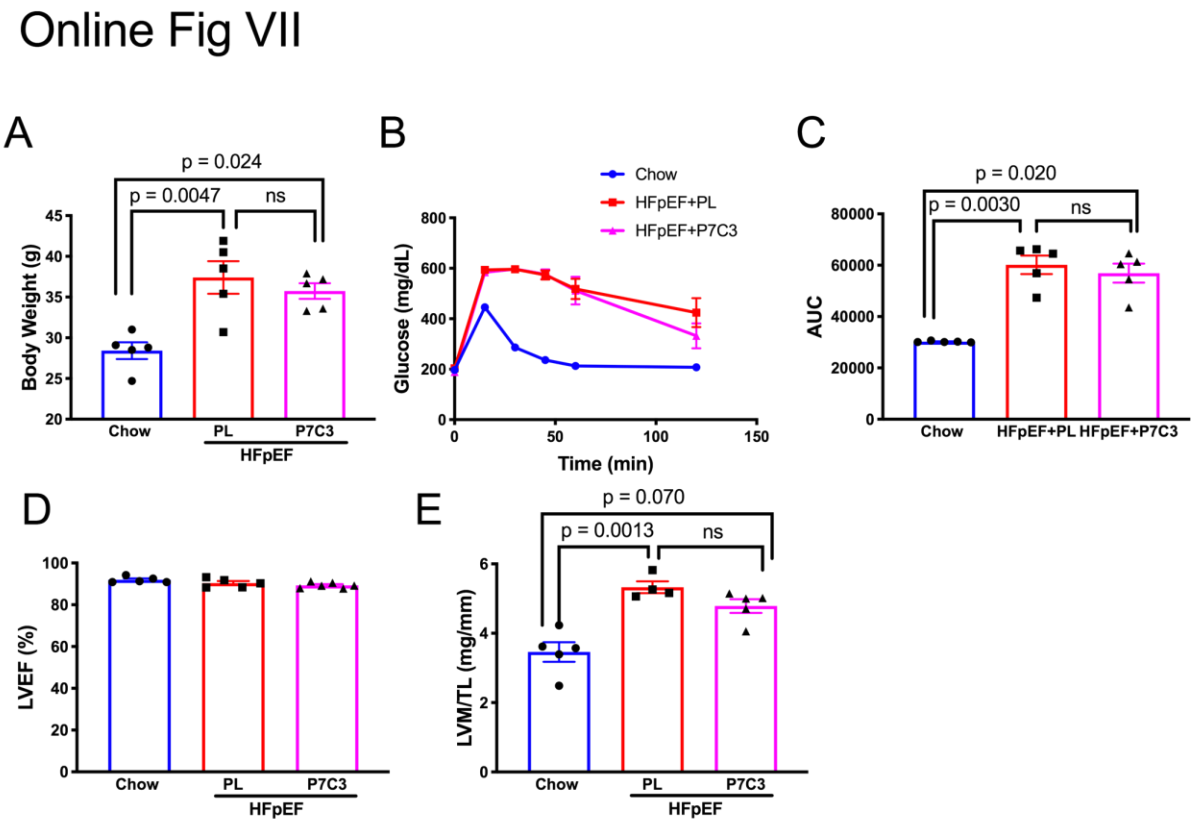
Online Fig VI



Online Figure VII. Phenotype of HFpEF Mice Treated with P7C3-A20

Mice were fed regular Chow (Chow) and HFpEF mice treated with placebo (HFpEF+Pl) vs P7C3 (HFpEF+P7C3-A20). N=5 mice per group.

(A) Body weight.
(B) Glucose levels during ipGTT test.
(C) Area under the curve of glucose tolerance test.
(D) LV ejection fraction (LVEF).
(E) Ratio of LV mass (LVM) to tibia length (TL).
Kruskal-Wallis test with Dunn's multiple comparisons test.



Online Table I. Clinical characteristics of control patients (non-HF) and those with HFpEF or HFrEF

	nonHF (n=15)	HFpEF (n=12)	HFrEF (n=15)
Demographics			
Age	51.8 ± 4.0	66.9 ± 3.4	50.4 ± 4.0
Female %	46.7	66.7	20
Ethnicity			
Caucasian %	80	25	73.3
African American %	13.3	66.7	26.7
Hispanic %	6.7	8.3	0
BMI	27.9 ± 2.1	38.6 ± 2.0	26.5 ± 1.3
EF %	61.7 ± 1.3	64.2 ± 1.7	17.1 ± 1.3
Medical history			
Hypertension %	40	100	100
Diabetes %	13.3	83.3	13.3
CAD %	6.7	16.7	20
CKD %	13.3	83.3	20
Atrial fibrillation %	6.7	33.3	46.7
Medications			
Diuretics %	0	100	100
ACEI or ARB %	26.7	66.7	73.3
beta blockers %	20	75	93.3
Insulin %	0	50	6.7

Online Table II. Echocardiographic parameters of Sirt3 mice

	αMHC-Cre, Sirt3$^{fl/fl}$ (Group A)	Sirt3$^{fl/fl}$ (Group B)	αMHC-Cre (Group C)
Conscious	n = 6	n = 6	n = 3
HR (bpm)	661 ± 14	658 ± 19	656 ± 21
LVID, d (mm)	2.6 ± 0.08	2.5 ± 0.08	2.5 ± 0.02
LVID, s (mm)	1.2 ± 0.06	1.1 ± 0.03	1.2 ± 0.02
IVS, d (mm)	1.1 ± 0.04	1.1 ± 0.03	1.2 ± 0.03
LVPW, d (mm)	1.1 ± 0.03	1.1 ± 0.06	1.1 ± 0.05
LVFS (%)	52.7 ± 1.2	54.1 ± 0.1	51.6 ± 0.4
LVEF (%)	89.3 ± 0.7	90.1 ± 1.1	88.7 ± 0.3
Sedated			
HR (bpm)	441 ± 12	406 ± 10	415 ± 9
E	683.9 ± 17.4	647.8 ± 19.1	672.0 ± 17.3
A	311.1 ± 41.3	343.4 ± 35.4	293.2 ± 26.5
E/A	2.8 ± 0.5	2.0 ± 0.2	2.3 ± 0.2
E'	13.8 ± 0.7 **#	19.1 ± 0.5	17.9 ± 0.2
E/E'	50.7 ± 3.7 **	34.3 ± 1.7	37.4 ± 0.6

Data are expressed as mean ± S.E.M. One-way ANOVA followed by Tukey's multiple comparisons test. **p<0.01 vs GroupB; # p< 0.05 vs GroupC

Online Table III. Echocardiographic parameters of NR/P7C3 vs placebo-treated mice

	Chow (Group1)	HFpEF+ Placebo (Group2)	HFpEF+ NR (Group3)	Effect Size (G3 vs G2) (95% CI)
Conscious	n=5	n=8	n=10	
HR (bpm)	719 ± 4	674 ± 13	697 ± 11	23.0 (-13.30-66.30)
LVID,d (mm)	2.34 ± 0.10	2.69 ± 0.09	2.7 ± 0.08*	0.01 (-0.27-0.35)
LVID,s (mm)	1.00 ± 0.04	1.20 ± 0.05 *	1.19 ± 0.03 *	-0.01 (-0.15 - 0.18)
IVS, d (mm)	1.10 ± 0.00	1.23 ± 0.4	1.23 ± 0.04	0.0 (-0.11-0.18)
LVPW, d (mm)	1.00 ± 0.05	1.19 ± 0.05 *	1.04 ± 0.02 #	-0.15 (-0.30-0.005)
LVFS (%)	56.97 ± 2.43	55.45 ± 0.66	55.25 ± 1.32	-0.20 (-5.19-3.91)
LVEF (%)	91.73 ± 1.33	91.12 ± 0.39	90.82 ± 0.86	-0.30 (-3.53-2.31)
Unconscious	n=8	n=7	n=10	
HR (bpm)	471 ± 13	456 ± 10	440 ± 10	-16.0 (-32.72-15.86)
E	578.40 ± 8.11	683.30 ± 24.96 *	576.0 ± 26.56 ##	-107.0 (-229.0 - 26.91)
A	387.60 ± 21.95	375.90 ± 26.16	339.60 ± 18.67	-36.0 (-141.20-49.26)
E/A	1.54 ± 0.11	1.86 ± 0.12	1.72 ± 0.07	-0.14 (-0.46-0.33)
E'	24.76 ± 0.93	15.86 ± 0.76 ****	19.17 ± 1.05 **	-3.31 (-1.95-8.35)
E/E'	23.60 ± 0.93	43.33 ± 1.40 ****	30.36 ± 1.16 ***###	-12.97 (-17.90 - -6.92)

	Chow (Group1)	HFpEF+Pl (Group2)	HFpEF+P7C3 (Group3)	Effect Size (G3 vs G2) (95% CI)
Conscious	n=5	n=5	n=6	
HR (bpm)	716 ± 16	689 ± 19	723 ± 22	34.0 (-19.16-96.76)
LVID, d (mm)	2.3 ± 0.1	2.5 ± 0.1	2.3 ± 0.1#	-0.2 (-0.55-0.07)
LVID, s (mm)	1.02 ± 0.04	1.12 ± 0.06	1.07 ± 0.03	-0.05 (-0.27-0.15)
IVS, d (mm)	1.04 ± 0.04	1.27 ± 0.03*	1.13 ± 0.09	-0.14 (-0.42-0.15)
LVPW, d (mm)	0.84 ± 0.05	1.10 ± 0.05**	1.13 ± 0.04**	0.03 (-0.20-0.32)
FS%	56.9 ± 1.2	54.4 ± 1.6	52.3 ± 0.7	-2.1 (-7.27-3.75)
EF%	91.9 ± 0.7	90.2 ± 1.0	89.1 ± 0.5	-1.10 (-4.19-2.53)
Unconscious				
HR (bpm)	453 ± 12	460±5.6	475.5 ± 11.5	15.50 (-48.63-75.13)
E	578.4 ± 12.3	709.1±7.0	603.8 ± 52.4	-105.30 (-243.40 - -26.33)
A	407.3 ± 18.4	389.1±48.7	367.9 ± 34.6	-21.20 (-125.50-166.60)
E/A	1.4 ± 0.05	1.9 ± 0.2	1.7 ± 0.3	-0.20 (-1.09-0.09)
E'	24.4 ± 1.3	15.3 ± 0.8 **	18.5 ± 1.4*	-3.20 (-5.14-9.36)
E/E'	23.9 ± 1.3	47.2 ± 3.3***	33.2 ± 2.7#	-14.0 (-31.35-5.00)

Data are expressed as mean ± S.E.M. One-way ANOVA followed by Tukey's multiple comparisons test.
*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. Group1;
# p<0.05 vs. Group2

Abbreviations: HR, heart rate; LVID,d, left ventricular internal diastolic diameter; LVID,s, left ventricular internal systolic diameter; IVS,d, end-diastolic interventricular septal wall thickness; LVPW,d, left ventricular end-diastolic posterior wall; LVFS, left ventricular fractional shortening; LVEF, left ventricular ejection fraction; E, peak Doppler blood inflow velocity across mitral valve during early diastole; A, peak Doppler blood inflow velocity across mitral valve during late diastole; E', peak tissue Doppler of myocardial relaxation velocity at mitral valve annulus during early diastole; HFpEF+PL, HFpEF mice treated with placebo; HFpEF+NR, HFpEF mice treated with NR, HFpEF+P7C3, HFpEF mice treated with P7C3-A20.